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UPTAKE AND DEPURATION RATES OF BULK ZnO POWDER, $ZnCl_2$, AND ZnO NANOPARTICLES BY *Eisenia fetida* BEFORE AND AFTER SOIL WEATHERING: THE POTENTIAL FOR TROPHIC TRANSFER

by

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A thesis

Presented to the faculty of

Towson University

In partial fulfillment

of the requirements for the degree

Master of Science

Department of Environmental Science

Towson University
Towson, Maryland 21252

December, 2013

TOWSON UNIVERSITY
OFFICE OF GRADUATE STUDIES

THESIS APPROVAL PAGE

This is to certify that the thesis prepared by: Megan E. Schulze

Entitled: "Uptake and depuration rates of bulk ZnO powder, $ZnCl_2$, and ZnO nanoparticles by *Eisenia fetida* before and after soil weathering: the potential for trophic transfer."

Has been approved by the thesis committee as satisfactorily completing the thesis requirements for the degree: Masters of Science

Chair, Thesis Committee	12/11/13 Date
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Committee Member	12/11/13 Date
Committee Member	Date
Committee Member	Date
Janet V. DeHaven Dean of Graduate Studies	1/2/14 Date

Acknowledgements

Foremost, I would like to thank my advisor Dr. Steven Lev for his encouragement, patience, and academic guidance through the duration of my master's thesis. His enthusiasm and astute insight has been invaluable personally and academically and I am exceedingly grateful.

I would like to acknowledge the support and profound input from my thesis committee members Dr. Ryan Casey and Dr. David Ownby. You were always receptive to my questions and helped to quell my anxieties of chemistry and ecotoxicology. The good advice and extensive statistical support of Dr. Joel Snodgrass must also be recognized. I cannot thank you all enough.

I also thank the staff from Johns Hopkins University for nanoparticle characterization through TEM and DLS analysis. Dr. Michael McCaffery, Dr. Howard Fairbrother, Erin Pryce, and Julie Bitter, I appreciate all of your efforts.

The unwavering support of my family, fiancé, and friends over my years in graduate school and through life in general has truly been my saving grace. I am especially thankful for my father who instilled a strong love for science and the environment in me from an early age, you're my inspiration. I love you all.

Lastly, this material is based in part upon work supported by the National Science Foundation under Grant Numbers CBE-NanoEHS 1236083. Without this funding I would not have been able to complete this research, thank you.

Abstract

UPTAKE AND DEPURATION RATES OF BULK ZnO POWDER, $ZnCl_2$, AND ZnO NANOPARTICLES BY *Eisenia fetida* BEFORE AND AFTER SOIL WEATHERING: THE POTENTIAL FOR TROPHIC TRANSFER

Megan Schulze

The earthworm *Eisenia fetida* was exposed to three treatments of isotopically-labeled zinc ($^{68}ZnCl_2$, bulk ^{68}ZnO, and ^{68}ZnO nanoparticles (NPs) at 148 mg/kg Zn) amended to topsoil for 21 days followed by 21 days in a "clean" topsoil to assess uptake and elimination rates (Initial). The exposure was repeated following a 21 day soil-aging period (Aged). All Zn treatments followed the same two-compartment elimination pattern. During the Initial experiment, bulk ^{68}ZnO demonstrated slower uptake in compartment one than $^{68}ZnCl_2$ and ^{68}ZnO NPs, then matched the rates of other treatments during the Aged experiment. A significant difference was observed in compartment two of the ^{68}ZnO NP treatment in both experiments. We saw no evidence of ZnO NP retention in *E. fetida* to suggest the potential for trophic transfer of ZnO NPs in a terrestrial food web. These data benefit risk assessment as ZnO NP (5-30nm) mimics $ZnCl_2$ in *E. fetida*.

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Chapter 1

Literature Review and Proposal

Introduction

The increasing use of nanoparticles in consumer products like washing machines, cosmetics and sunscreens, raises the question as to the fate of the nanoparticles (NPs) in the environment (Binion 2008). As an example, ZnO and TiO_2 NPs have the ability to absorb ultraviolet (UV) light when below the size of 100 nm, and are commonly found in sunscreens. These particles can be released into the environment via wastewater treatment systems, stormwater retention ponds, or soil and surface water systems in all phases of their life cycle (Botta et al., 2011). Environmental exposure to nanoparticles is not a new concept and organisms have adapted to natural exposures; the concern lies with the potential release of manufactured nanoparticles that are engineered with surfactants, polymers, impurities, and coatings, and may have a detrimental effect on the environment (Unrine et al., 2008).

Depending on the source product containing manufactured nanoparticles, NPs can enter the environment through landfills, incineration, leaching through sewage treatment plants, soil and surface waters (Gottschalk et al., 2009). Specifically, Gottschalk et al. determined that nano-ZnO's most prominent pathway into the environment was flows from manufacturing and production plants to sewage treatment plants, while TiO_2 and Ag NPs overwhelmingly entered natural systems through stormwater treatment influent, and flow from manufacturing and production plants to landfills. All three metal NPs were similarly introduced into the terrestrial environment via sewage sludge application to soil; the U.S. practices direct land application with about 63% of its sewage sludge (2009).

The modeling efforts by Gottschalk et al. (2009) reveal that overall, TiO_2 is likely to be the most abundant metal NP found in U.S. sediments and sludge treated soils followed closely by nano-ZnO. Gottschalk et al. (2009) show that the levels of metal NPs in natural environments are likely to keep increasing as nanotechnology continues to expand – making research on nanoparticle fate in the environment critical.

Brar et al. (2010) discussed the fate of carbon-based nanoparticles in wastewater and identified the potential for NPs to bind with particulate organic matter or other suspended solids in the wastewater as a serious issue. If a water treatment plant does not remove these nano-bound contaminants, they could be directly released into the environment (Brar et al., 2010). The expectation is that through the wastewater treatment process, most of these NP agglomerates will settle out into sewage sludge. This sludge, depending on local regulations, can be disposed of, spread on agricultural fields, forests, range lands, or on land in need of reclamation as an organic additive, thus creating a pathway for NPs into the terrestrial ecosystem (Brar et al., 2010 and Li et al., 2007). In the United States, 16,000 wastewater treatment plants generate approximately 7 million dry tons of sewage sludge, and almost 60% of that sludge is applied to land (Li et al., 2007). While the effectiveness of the individual treatment plant to flocculate particulates in the waste stream will impact the amount of NPs that are discharged to surface waters through effluent flow, or land via sewage sludge, this is a potential pathway that requires more investigation. With the majority of NPs being released through aqueous and sewage sludge mediums, aquatic and terrestrial systems are a likely sink for manufactured nanoparticles (Unrine et al., 2010). The fates of these nanoparticles once released into the

environment and the potential for trophic transfer, in the environment, are open questions.

Potential for trophic transfer of NPs in terrestrial systems

In terrestrial systems in particular, the potential for trophic transfer of NPs rather than the ionic metal derived from the dissolution of the NPs has not been adequately addressed for soil-dwelling organisms. Recently, Judy et al. (2011) were able to present convincing evidence for the trophic transfer and biomagnification of Au NPs from the tobacco plant *Nicotiana tabacum* L. cv *Zanthi* to the tobacco hornworm *Manduca sexta*. Judy et al. (2011) used a variety of analytical methods to track the uptake, accumulation and trophic transfer of Au NPs including bulk digestion of tissues and analysis by ICP-MS, Laser Ablation ICP-MS, and micro-X-ray fluorescence to map and identify Au NPs that have been transferred to the primary consumer as opposed to adsorbed onto tissue surfaces or incorporated as solubilized Au ions derived from NP dissolution.

Earthworms directly process soil through their digestive system making them susceptible to changes in the chemistry of the soil environment. As they are an important food source for other terrestrial organisms, earthworms may represent a critical entry point into the food web for trace metal pollutants, including NPs, in terrestrial systems (Beyer and Cromartie, 1987; Pizl and Josens, 1995 and references therein). Therefore, it is important to investigate the possibility that earthworms may provide a trophic transfer pathway for NPs in terrestrial systems. Earthworms can be exposed to NPs through dermal contact or through ingestion of contaminated materials. A study by Unrine (2010) found evidence that intact Au nanoparticles were not only ingested by earthworms, but distributed throughout the body tissues after exposure; the highest level of Au being

found within the gut tissues after purging. This raises speculation about nanoparticle accumulation within the food chain. Commonly identified nanoparticles in soil environments include carbon (C60), ultra-fine titanium oxide (TiO_2), silver (Ag), and zinc oxide (ZnO) (Handy et al., 2008, Navarro et al., 2008, and Hu et al., 2010).

A more recent study focused on trophic transfer of TiO_2 NPs because of their growing abundance in the agricultural market; i.e. extensive use in fertilizers and therefore direct, and often unregulated, land application (Yeo and Nam, 2013). In Korea, rice paddy fields are commonly fertilized with material containing manufactured TiO_2 nanomaterials (nanoparticle and nanotubes (NTs)) knowing that these materials are efficient at absorbing light and enhancing photosynthesis while simultaneously promoting Rubisco activase, an enzyme essential for carbon fixation in plants (Nair et. al., 2010). This study focused on the food web interactions within a rice paddy ecosystem, including compartments for environmental media, plants, and lower and higher trophic organisms over 17 days. Yeo and Nam's research found a high level of nano-TiO_2 concentrated in the roots of the water dropwort (*Oenanthe javanica*), lower biofilm, and algae (*Spirogyra* sp.) suggesting that nanomaterials that are added into an aquatic environment at the surface, can migrate and accumulate in lower trophic levels. A 200% (NPs) and 500% (NTs) increase of nano-TiO_2 was observed in the parasitic nematode (*Meloidogyne* sp.) when feeding on the water dropwort. Also, a high level of nanomaterial transfer occurred from the plankton that consume biofilm to the ricefish (*Oryzias sinensis*). Most of the trophic transfer of nano-TiO_2 was seen in lower trophic levels probably due to nanomaterial agglomeration and settling, but the ability for

nanomaterials to reach higher trophic levels in aquatic system is possible and should be thoroughly researched in terrestrial systems as well (Yeo and Nam, 2013).

While the trophic transfer data provided by Judy et al. (2011) strongly suggests the potential for the trophic transfer of NPs in terrestrial systems, the Au NPs in that study were transferred to the primary plant consumer through hydroponic suspension thereby excluding a soil medium from the consumer web. To provide a more realistic view of nanoparticle movement through a terrestrial food chain, Unrine et al., 2012 investigated the transfer of Au NPs from soil, to earthworm consumer, to vertebrate consumer. Earthworm *Eisenia fetida* and bullfrog *Rana catesbeiana* were chosen respectively as food web consumers. To distinguish between trophic transfer uptake and direct exposure uptake, one group of *R. catesbeiana* were fed *E. fetida* with Au NP amendment, and another group was exposed via oral gavage of Au NP solution. Results showed that *R. catesbeiana* had an overall higher body burden of Au NPs in the trophic transfer group as compared to oral gavage, indicating that metal NPs may become more biologically available as they move through a food chain (Unrine et al., 2012). Unrine et al. also dissected and analyzed individual organs within the previously exposed bullfrogs to ensure that Au NPs were in fact absorbed by the bloodstream and transferred throughout the body to different tissues and not simply adsorbed to the exterior of the gut (2012). The highest Au concentrations were found in the spleen, kidney, and liver respectively. Specifically, the highest Au concentrations from the trophic transfer group were found in the liver and kidneys, demonstrating that the Au NPs traveled through the blood stream and settled within these organs (Unrine et al., 2012).

Brayner et al. (2006) demonstrated the effect of ZnO NPs on *Escherichia coli* and found that the ultrafine particles (~7-15 nm) resulted in complete mortality to the prokaryotes at concentrations between 10 and 3 mM. Specifically, Brayner et al. found that as the ZnO NPs came in contact with the bacteria, their cellular membrane became damaged allowing the nanoparticles to enter the bacteria. To expand on the bacterial cell death characterized in the Brayer experiment with *E. coli*, Reddy et al. (2007) investigated the effect of ZnO NPs on *E. coli* (gram-negative), *Staphylococcus aureus* (gram-positive), and human immune cells as they are our first line of defense against pathogens. Reddy found the inhibition of cell growth in bacteria to be >3.4 mM for *E. coli*, and >1 mM for *S. aureus* when exposed to ZnO NPs (~13 nm). In contrast, the human T cells became only slightly inhibited at >5 mM with many cells remaining active at >10 mM. The Reddy et al. (2007) experiment shows that ZnO NPs appear to have differential toxicity depending on the type of cell, eukaryotic or prokaryotic, at specific concentrations. These studies indicate an important place for ZnO NPs in nanomedicine, but above lab-controlled concentrations there is a risk of increased human cell death. Over the broad spectrum, however, the success of metal oxide NPs as an anti-microbial raises speculation about the interaction of NPs with important microbial soil species used for commercial purposes, such as agriculture. Land use application of metal oxide-contaminated sludge could unintentionally impact soils that depend on microbial activity for success. Most importantly, as zinc nanoparticles become more common in the environment their ecological risks and potential toxicity to organisms and microbes must be better characterized.

Evaluating the fate of Zn NPs in the terrestrial environment is a challenging proposition given the status of Zn as a micronutrient for many terrestrial macro fauna. Earthworms are one of several key soil-dwelling organisms used in terrestrial risk assessment because they directly process soil through their digestive system making them susceptible to changes in the chemistry of the soil environment. Like many other soil dwelling organisms, Zn is an essential micronutrient for earthworms and is physiologically regulated (Kamitani and Kaneko, 2007 and references therein). The typhlosole is a high surface area (~2000-5000 μm^2) region of the intestine and is associated with the highest density of chloragosomal cells that line the exterior surface of the intestine. These cells function as an accessory digestive gland and the site of glycogen synthesis, deamination of proteins, and production of ammonia and urea. The typhlosole and chloragosomal cells may function as the primary regulation point for micronutrients and other metals (Morgan, 1984; Morgan et al., 2004 and references therein). The exposure response of an organism to a micronutrient will be uni-modal with the health of the organism being stable over a range of optimal micronutrient concentration conditions and poor in conditions that are micronutrient limited or where there is an excess. As a result, a number of studies have noted a lack of correlation between earthworm Zn body burden and Zn exposure concentration in traditional exposure experiments where only whole body analysis is done (Van Gestel et al., 1993; Spurgeon and Hopkin, 1999; Lock and Janssen, 2001; Lanno et al., 2004; van Straalen et al., 2005).

Several approaches have been suggested to overcome this complication and more accurately track the uptake and storage of Zn by earthworms. In a set of experiments

using a well characterized field soil amended with roadway dust, Lev et al. (2008) documented the uptake of roadway-derived Zn by *Lumbricus friendi* over a range of Zn concentrations meant to bracket the observed concentration range in stormwater retention basin soils (46 to 460 mg kg^{-1}). Lev et al. (2008) used a combination of dissected tissues (i.e. gut tissue vs. whole body) and synchrotron mapping to document the relationship between the gut tissues, specifically the typhlosole, and Zn exposure concentration along with the presence of saturable uptake kinetics in those tissues. In this experiment, earthworm total body residues at the end of a 28 day exposure period were not significantly correlated with soil Zn concentrations. Micro-Synchrotron X-ray Fluorescence (μSXRF) and Zn elemental maps of cross-sections from the section of the body and the intestine show the Zn concentration of the typhlosole region of the intestine to be correlated with the level of Zn exposure. This was confirmed by a statistically significant positive linear relationship between Zn concentration (direct chemical measurement) in the gut tissues and Zn exposure concentration.

The use of the Critical Body Residue (CBR) or total body burden approach to assess bioaccumulation and the potential for trophic transfer is a common tool in risk assessment. This approach homogenizes information on the compartmentalization of metals and obscures information on the potential for trophic transfer (Vijver et al., 2004 and references therein). For example, metals taken up by an earthworm will be compartmentalized into a number of different organs, tissues and metal-rich granules. The availability of metals sequestered in these different compartments may vary and will impact the potential for trophic transfer to a predator (Vijver et al., 2004). There are six internal metal compartments that have been identified as important to describing metal

accumulation; 1) granules, 2) lysosomes, 3) organelles and microsomes, 4) nuclei, cell membranes, tissues and intact cells, 5) heat denatured protein and, 6) heat stable proteins (Vijver et al., 2004 and references therein). Of these sub-cellular compartments, Vijver et al. (2004) view 1 and 6 as important for uptake, 2 and 3 for elimination and all but 1 as important for trophic transfer. In 2006, Vijver et al. examined the subcellular fractions of the earthworm *Aporrectodea calignosa* after exposure to spiked field soils and found that these fractions provide reliable and understandable information on uptake and accumulation of metals in these organisms that might otherwise be lost when using the CBR approach.

While it is clear from this brief review that interpretation of the bioaccumulation patterns and effects of regulated metals on earthworms is challenging without carefully examining the body fractions most closely associated with metal uptake and sequestration, neither of the approaches described can track the fate of an anthropogenic, regulated metal in the environment. This is a critical question for the risk assessment associated with engineered NPs. Rogers et al. (2010) presented a chemical method for examining soil Zn bioavailability and the relative magnitude of the soil Zn pool interacting with the tissues of the earthworm *L. terrestris*. They used a stable isotope approach to quantify the turnover rate of the enriched stable isotope ^{68}Zn in three different earthworm tissue compartments and related that rate to bioavailability of Zn in the soil. Rogers et al. (2010) used the dilution of a stable isotope tracer as a way to evaluate initial uptake rates. In their investigation, earthworms were equilibrated in a ^{68}Zn-spiked background soil, so that their tissues displayed an enriched ^{68}Zn/^{66}Zn ratio relative to the ^{68}Zn/^{66}Zn found in nature (i.e. ~0.67). The isotopically-labeled worms

were then introduced to exposure soils that were amended with different levels of $ZnCl_2$. The subsequent ^{68}Zn dilution rates derived from the organ tissues were found to be positively correlated with Zn content in the soil providing a chemical tool for the evaluation of soil Zn bioavailability and a method to track isotopically distinct pools of a regulated metal like Zn in *L. terrestris*.

In addition to the difficulties associated with tracking the fate of Zn NPs in soil systems there are also open questions about the toxicity, bioavailability and, long-term fate of Zn NPs as compared to other forms of Zn (i.e. bulk powder and $ZnCl_2$) in soil systems. The small particle size and high surface area of nanoparticles create the potential for adverse effects on organisms both through the release of metal ions from these particles but, also through the direct uptake and incorporation of the particles into individual cells within the organism. There have been few studies examining the effect of weathered metal oxide nanoparticles on terrestrial invertebrates. Because manufactured nanoparticles have many different coatings and surface features, it is likely that soil biotic and abiotic processes will change their surface chemistry thereby altering bioavailability and soil interactions as they age (Unrine et al., 2008).

A concern in weathered soils containing metal nanoparticles, especially ZnO, is the release of metal ions (Kool et al., 2011, and references therein). In a soil exposure test with ZnO NPs and the nematode *Caenorhabditis elegans*, Wang et al. (2009) demonstrated that toxicity could be attributed to the dissolution of the entire particle and release of free Zn ions into solution. Kool et al. (2011) had a similar result when they exposed the springtail *Folsomia candida* to bulk ZnO powder, ZnO NPs and $ZnCl_2$ in a field soil. After a 4-week exposure, they concluded that the toxic effects observed were

the result of Zn ions derived from NP dissolution during the exposure. Unrine et al. (2008) suggests that NP size has a direct effect on bioaccumulation because as nanoparticles age in soil, they are likely to agglomerate in soil pore water. Nanoparticles smaller than 10 nm are more easily absorbed in the gut and transferred throughout the body's tissues than NPs that are >100 nm in radius (Unrine et al., 2008). In a study comparing the uptake of Au nanoparticles in earthworms, the worms were exposed to different Au treatments (Au ions, 4, and 18 nm size NPs). After examining the post-exposure tissues using laser ablation-inductively coupled mass spectrometry (LA-ICP-MS), results confirmed that uptake was dependent on particle size showing the highest uptake from the dissolved Au ion exposure then 4 nm and 18 nm Au NP exposures respectively (Unrine et al., 2008 and references therein).

In this investigation, we propose to use isotopically-labeled ^{68}ZnO nanoparticles to track the fate of NPs in soil and the potential for uptake and trophic transfer using the earthworm *Eisenia fetida*. *E. fetida* is a standard organism for soil risk assessment and has demonstrated excellent survival under the control conditions to be used in this work. The isotopically-labeled NPs we have synthesized for use in the proposed experiments were generated from a modified reflux method as described by Dybowska et al. (2011) and Chieng et al. (2012). The behavior of the ^{68}ZnO NPs was compared with the uptake and elimination rates of non-nano bulk ^{68}ZnO powder and $^{68}ZnCl_2$ to illustrate the behavior of ionic versus particulate (Zn NPs and ZnO powder) forms of zinc in a soil system with *E. fetida*. The amended soils were split prior to introducing earthworms and one soil set was be kept at constant moisture and temperature for 21 days before introducing earthworms to evaluate the impact of soil ageing on the bioavailability of the

different Zn forms. We used this general approach to test the following null hypotheses; 1) $^{68}Zn/^{66}Zn$ isotope ratio in *E. fetida* after an uptake and elimination period are the same for all three forms of zinc, indicating that zinc is easily eliminated by *E. fetida* regardless of form and, 2) there is no difference in uptake or elimination rates after 21 days of ageing among the three zinc treatments and that these treatments are not different from the initial experiment. These relationships are presented in Figure 1 along with a model for an alternate outcome where there are different rates of uptake and elimination either between the different forms of Zn in either experiment 1 or after ageing.

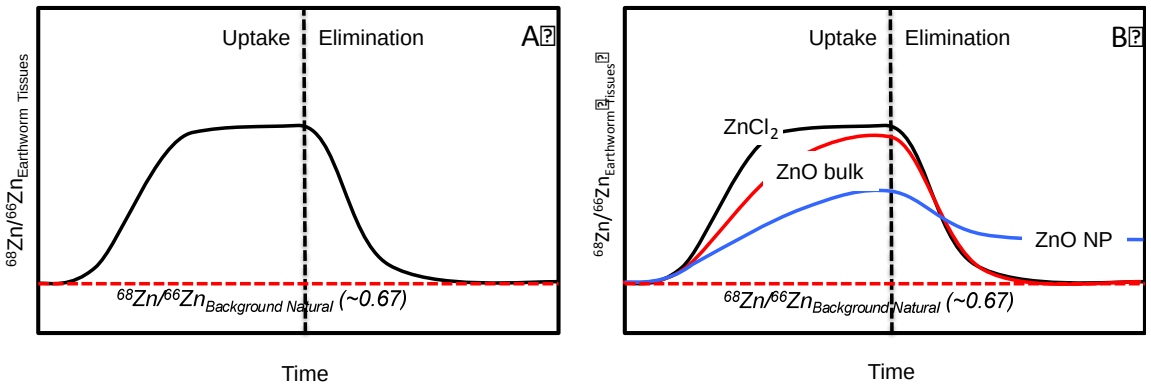


Figure 1. The model presented in (A) is a representation of the isotopic behavior predicted in both hypothesis 1 and 2. The black curve represents the expected pattern of uptake and elimination if there is no difference in the availability of the three Zn species nor is there any effect on availability after ageing. The curves presented in (B) represent one possible outcome if hypotheses 1 and 2 prove to be incorrect. In this model there are different availabilities among the three phases during uptake and Zn associated with the NPs cannot be completely eliminated from the organism. This would suggest that ZnO NPs are being retained as discrete particles since ionic Zn appears to be labile, using the $ZnCl_2$ elimination curve as a proxy for ionic Zn.

Chapter 2

Pilot Tests

Introduction

A series of pilot experiments were preformed prior to the definitive tests to establish optimal soil and Zn concentrations to ensure maximum earthworm survival over the experimental period. In all experiments described below, adult earthworms of the species *E. fetida* were used. All worms were purchased from Carolina Biological Supply Company and analyzed for background levels of Zn and Zn isotopic composition prior to use. The worms were used in pilot tests using non-isotopic Zn and in two definitive tests using non-isotopically labeled Zn. .

Experimental approach

Before definitive tests could be carried out, we conducted two pilot range-finding experiments to evaluate toxicity and soil type. Pilot tests were run without ^{68}Zn isotope. Because this experiment was designed to run over a 42-day exposure period, we needed to confirm that *E. fetida* could survive the duration of the experiment in the soil type and at the selected level of zinc exposure. Standard toxicity testing methods were expected to be used to determine the EC_{50} for each form of Zn previously described and these values will be used to set the optimum concentration for the definitive experiments. Non-isotopically labeled Zn materials for these experiments were purchased from Alfa Aesar. Before these range-finding tests, we compared the use of OECD-type soil versus an in-house top soil to find the most beneficial habitat for *E. fetida*. The concern with using an artificial OECD-type soil is that the earthworms may not be supplied with enough organic material (OECD soil is only comprised of 10% peat) to sustain them for 42 days

without manual feeding (OECD, 2000). Manual feeding would have added the risk of mold and likely reduce soil intake by the test organisms, which could alter final results. Top soil is rich in organic matter and may offer a better medium for the worms. Previous toxicity tests comparing ranges of pH and organic matter in OECD soil to the survivability of *E. fetida* when exposed to zinc, found that soils with the lowest pH and lowest amount of organic matter made zinc more soluble and therefore more bioavailable and toxic to the earthworms (Spurgeon et al., 1996). With this knowledge in mind, pilot tests were important to determine the most appropriate soil medium for the earthworms.

Pilot test – soil type

In the soil medium test, 24 adult *E. fetida* were individually placed into 50 mL test tubes containing ~40g of two different soil types at 56% moisture content (~50% of the water holding capacity of the soil). Twelve worms were placed in laboratory-prepared, OECD-type soil (70% sand, 20% clay, 10% peat) and the remaining 12 were placed in a commercially available top soil. All worms were held in test tube racks in an incubator at a constant moisture and temperature (18° C) with 12 hour light and darkness cycles for 42 days to simulate the duration of the definitive experiment. End points of weight change and mortality were recorded on days 0, 21, and 42.

Pilot test – range-finding

Based on the results of the soil type pilot test, the most beneficial soil type, top soil, was amended with 14 different concentrations of non-isotopic Zn (0, 100, 200, 500, 900, 1000, 1200, 1400, 1600, 1800, 2000, 3000, 4000, and 5000) using three different forms of Zn ($ZnCl_2$, ZnO bulk powder, and ZnO NPs), with 6 replicates of worms per each time point. A 6 x 14 x 3 design was used for this experiment. A set of clean control

soils were included at this stage to evaluate weight change. One worm was placed in each 50 mL test tube containing ~40g of the treatment soil type at 56% moisture content (~50% of the water holding capacity of the soil). Worms were exposed to all concentrations for 21 days to represent the uptake phase of the definitive experiment; there was no need to extend this pilot test to the full 42 days because after day 21 in the definitive experiment the worms are moved into "clean" soil causing minimal stress. All worms were held in an incubator at a constant moisture and temperature (18° C) with 12 hour light and dark cycle. End points of weight change and mortality were recorded at days 0 and 21. All individuals were randomly assigned positions and the experiment was blocked by shelf and position on the shelf (front vs. back) within the incubator. After removal of all surviving worms from the treatment soil on day 21, each worm was sacrificed via methanol exposure, briefly stored in 10% formalin, and cleared of gut contents by dissection. During dissection, each earthworm was cut anterior to posterior and washed with E-pure water multiple times to remove all entrained soil. Following dissection, worms were dried, weighed, digested and analyzed by Inductively Coupled Plasma Mass Spectrometry (ICP-MS, see details below).

Chemistry and analysis

Amended soils at each of the 14 exposure concentrations for each Zn treatment were analyzed using acid extractable digestion methods (EPA method 3050B). Acid digestions of earthworms from pilot tests were performed in Teflon vials (~15 mL) using trace metal grade 7N HNO_3 and H_2O_2 at 120°C for 12 hours in a hot plate digestion block. All worm and soil samples were analyzed using ICP-MS.

We used an asymptotic model to investigate the relationship between nominal concentrations and extractable soil Zn concentrations:

$$y = \frac{a}{1+ b^{-cx}} \quad (1)$$

In this model, a is the asymptote. To compare asymptotes between soil amendments, we constructed 95% confidence intervals for a. We used a t-test to test the null hypothesis that model parameter estimates did not differ from zero for all parameters in the model. Figure 5 is a plot of the acid extractable concentration of Zn in each treatment soil versus the nominal values and Table 1 lists the values from the model output.

Results

Results from the pilot experiments are presented in Tables 1-3 and Figure 2-6. Figure 2 is a plot of weight change versus time from the first pilot test of survivability of *E. fetida* in top soil and lab-prepared OECD soil over the course of 42 days. There were apparent differences in the observed weight change for earthworms in each soil type with worms exhibiting weight gain in the topsoil treatment. Figures 3 and 4 show the data from the range-finding test to see which concentration of Zn would result in the highest uptake while causing the least stress (weight loss) in *E. fetida* over the 21 day uptake phase. Figure 3 is a plot of weight change versus exposure concentration for each Zn treatment. Figure 4 is a plot of Zn body burden for *E. fetida* over the 21-day exposure period for each Zn treatment.

Figure 6 is a plot of earthworm survival versus exposure concentration in each treatment. We used a logistic regression model and analysis of deviance to investigate differences in earthworm survival among soil amendment types and concentrations. We

included an interaction term in the model. We used Fisher's exact test to compare survival among amendment types at individual nominal concentrations. Survival differed significantly among amendment types at the highest concentration, 5000 mg/kg Zn (P = 0.021), but not at concentrations lower than 4000 mg/kg Zn (P > 0.050).

Table 1. Model results Acid-Extractable Zn in Top Soil

Parameters	**Estimate**	**Std. Error**	**T value**	**Pr(>|t|)**	**95% CI**	
$ZnCl_2$					**lower**	**upper**
a	2539	110.4	22.99	1.19E-10	2323	2755
b	34.37	9.121	3.768	0.00311		
c	0.0015	0.0002	9.466	1.28E-06		
Bulk						
a	3386.	329.8	10.267	5.68E-07	2740	4032
b	11.44	2.181	5.247	0.000274		
c	0.0010	0.0001	6.397	5.10E-05		
Nano						
a	3820	273.1	13.987	2.37E-08	3285	4355
b	17.24	5.725	3.012	0.01183		
c	0.0013	0.0002	5.825	0.000115		

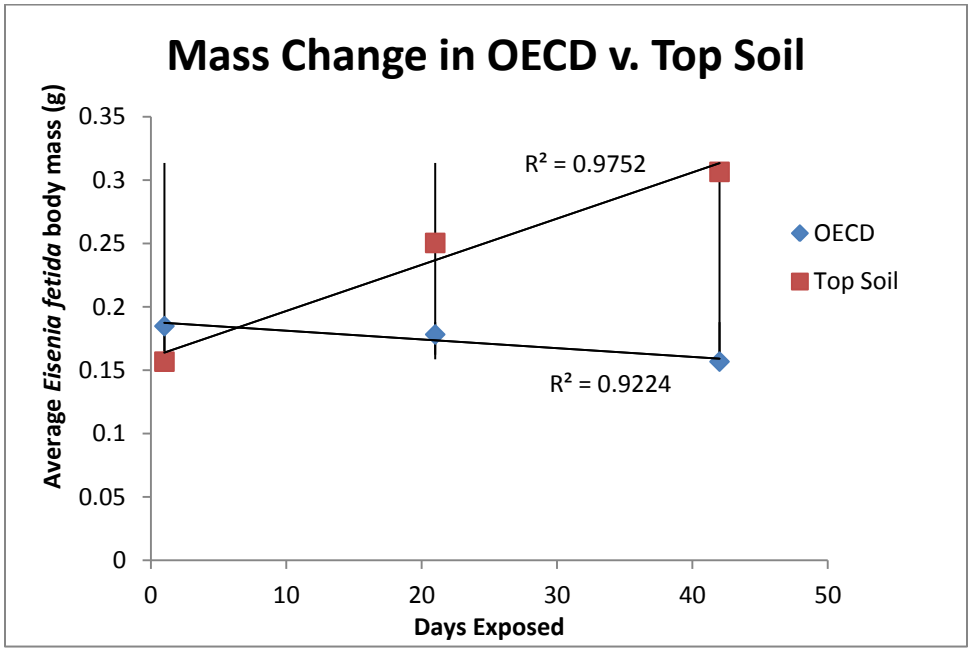


Figure 2. Comparison of *E. fetida* body mass change over 42 days in OECD soil and top soil. Error bars represent standard deviation among samples.

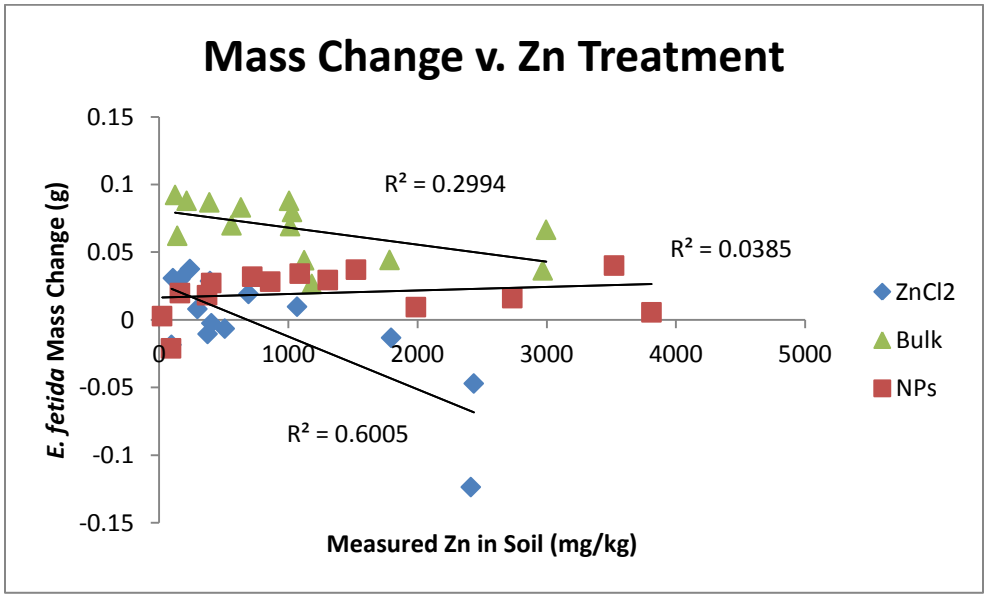


Figure 3. Effects of Zn concentrations on *E. fetida* body mass over 21 days.

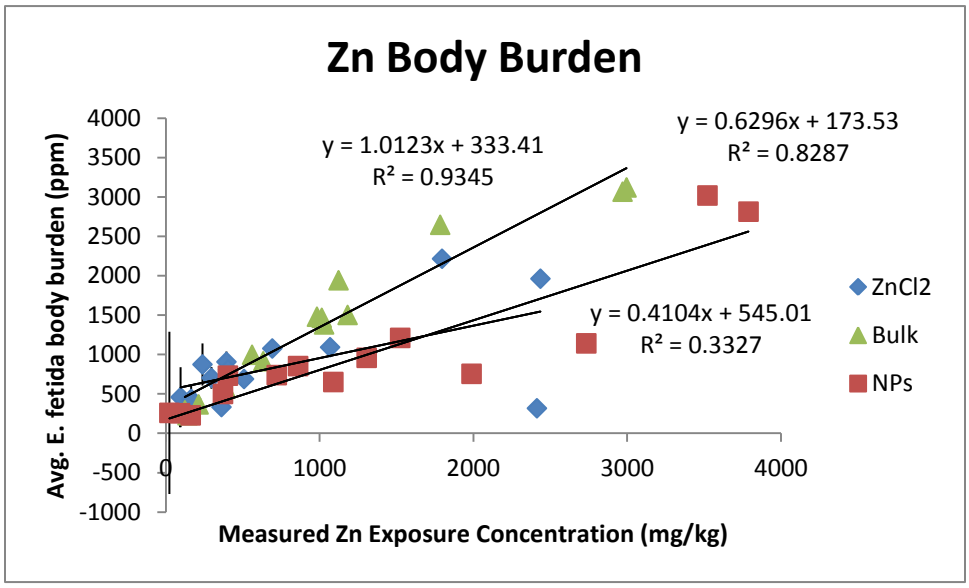


Figure 4. Overall Zn body burden in *E. fetida* over 21 day range-finding pilot test.

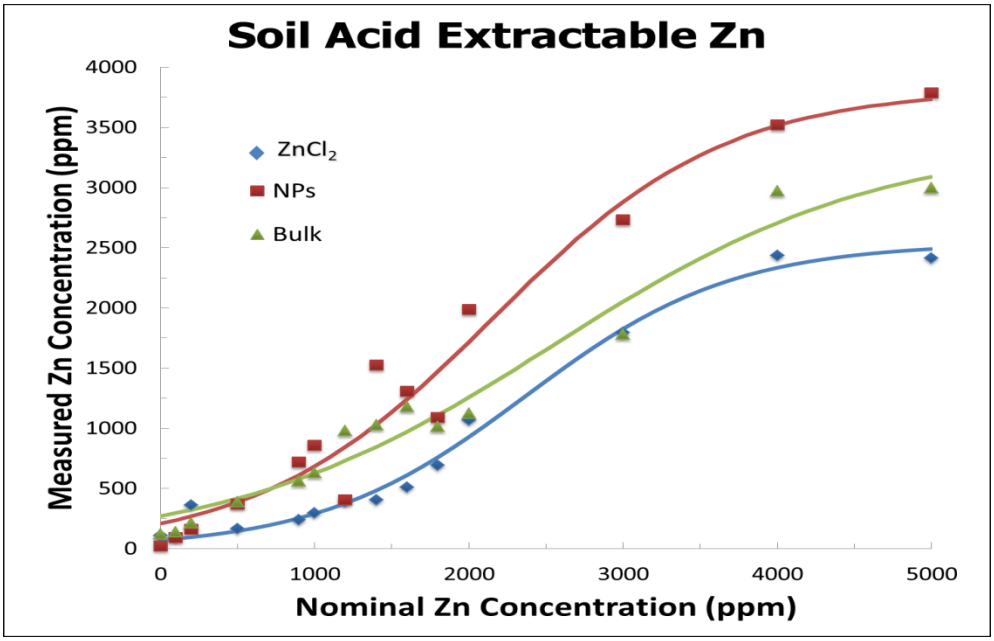


Figure 5. Acid extractable Zn retrieved from test soils in the range finding pilot test.

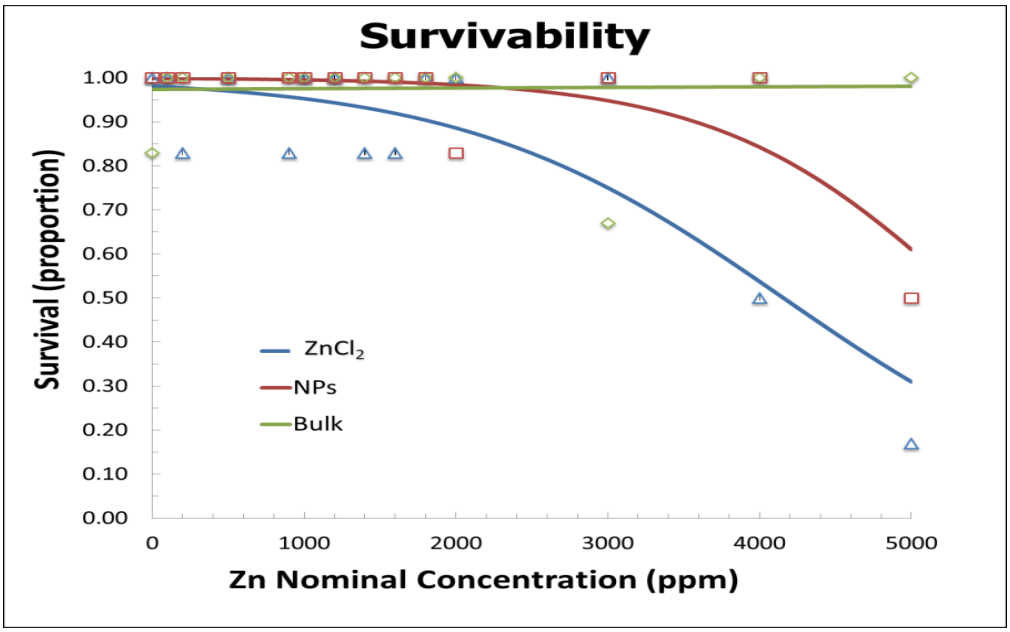


Figure 6. The survivability curves showing how *E. fetida* responded in each Zn treatment over all concentrations.

Discussion

Based on the results of the survivability experiment, top soil was selected as the best medium for the rest of the experiments. By day 21, earthworms in the top soil treatment weighed, on average, 40.5% more than earthworms from the OECD soil. By day 42, earthworms in topsoil weighed, on average, 95.4% more than those in OECD soil. Topsoil resulted in marked improvement of earthworm growth and both soils exhibited identical limited mortality rates (Figure 2). These results were consistent with van Gestel et al., (1992) in that *Eisenia sp*. are typically found in organically enriched soils, like manure, and may not be physiologically able to gain nutrition from artificial soil media like an OECD-type soil.

Earthworms in the $ZnCl_2$ treatment exhibited the most weight loss likely due to the high bioavailability of Zn in the ionic form. These data agreed with the survivability curve presented in Figure 6. One possibility for the lower uptake in the $ZnCl_2$ treatment could be slower feeding rates due to exposure stress evident as weight loss and the higher mortality rate (Figure 3 and 6). The survivability data suggested that all three treatments were significantly different from one another (P value = 0.003219). The highest Zn body burden was observed in the bulk treatment, followed by NPs with $ZnCl_2$ treatment worms exhibiting the least uptake (Figure 4). The high body burden measurements in the bulk treatment could be attributed to the larger particles entering the earthworm, remaining bound in the particulate form due to lower surface area, and therefore not being as easily regulated as Zn^{2+} in the ionic form ($ZnCl_2$, some ZnO NPs). The small particle size, high surface area and surface activity of nanoparticles, in general, led us to believe that the ZnO NP treatment could have acted differently that the other two treatments. Perhaps the NP treatment would have released free Zn^{2+} from the particle and resulted in a stressful environment for the earthworms as seen in the $ZnCl_2$ treatment. Another option would be for the Zn to remain bound within the particle, but assimilated into tissues faster because of the small particle size, and accumulate at higher levels of body burden. In these pilot tests the NP treatment fell somewhere in the middle of the three Zn treatments in relation to body burden and weight loss (stress) to *E. fetida*. The nanoparticle treatment was, however, the most readily extracted from the soil in the acid extractable analysis.

The target concentrations for each soil treatment group were based on nominal values from 0-5000 mg/kg total Zn (Kool et. al., 2011). The measured acid extractable concentrations deviated from the nominal within each amendment type and between

amendments. The measured exposure concentrations ranged from 0-3900 ppm Zn and were likely related to changes in the form of the Zn after amendment as it interacts with the soil. It is also possible that acid extractable methods were not able to recover all of the amended Zn.

Conclusions

By conducting a series of pilot tests, we could confidently move forward with the optimal soil medium and Zn concentration to provide the most advantageous environment for survival of *E. fetida* during the definitive experiments. The earthworms experienced the most weight gain in top soil rather than in OECD, lab prepared soil. As a result, top soil was selected for the definitive experiments. The Zn exposure concentration for the definitive experiment was set in the range of 100-300 mg/kg Zn for the definitive experiments. At that level of exposure, all Zn treatments exhibited an increasing body burden without notable weight loss (Figures 3 and 4). Due to higher than expected survival rates it was not possible to calculate an LC_{50} or LC_{10}.

Chapter 3

Definitive Experiments

Introduction

Increasing use of nanomaterials, dimensions of 1-100 nm, in many fields of manufacturing (cosmetic, solar voltaics, rubber, paints, etc.) has expanded the potential for release of metal nanoparticles (NPs) into the environment. Specifically, manufactured ZnO and TiO_2 NPs are known for their light absorbing and light scattering properties and are major components of sunscreens and are some of the most popular metal nanoparticles produced and studied (Popov et al., 2011, Ma et al., 2013). Metal nanoparticles can enter the environment through many different pathways: landfills, incineration, leaching through sewage treatment plants, and soil and surface waters (Gottschalk et al., 2009).

Toxicological effects of metal and metal oxide nanoparticles are important areas of study for three major reasons: the potential release of free metal from the nanoparticle, their ability to interact with, and produce harmful substances from, surrounding media (adhesion), and the potential to adversely interrupt biological processes at the molecular level (Ma et al., 2013). In the case of terrestrial environments, nanoparticles tend to agglomerate, or cluster together. Agglomeration increases persistence due to lower surface area and lower theoretical equilibrium solubility; this persistence can lead to the potential to enter food webs through uptake in lower trophic species (Borm et al., 2006).

Earthworms are commonly used as bio-indicators in contaminated soils studies because of their natural abundance (comprising 60-80% total soil biomass), sensitivity, ease of laboratory handling, and presence at the base of most terrestrial food chains

(Bouché, 1992). In the case of Zn nanoparticles, worms are able to regulate and/or sequester metals based on the abundance in a soil in an attempt to detoxify free metal ions. As a result, tissue body burdens can be used to express uptake and elimination rates (Sheppard et al., 1998) for manufactured nano-metals entering terrestrial pathways. Identifying bioavailability of metal nanoparticles through uptake and elimination kinetics in a soil environment is difficult because of the heterogeneous nature of soils but an important tool none the less in order to better understand charged particle interactions within the biotic ligand model (BLM) (Vijver et al., 2006). The presence of soluble metals like Zn found in pore water are a major contributing factor for metal uptake in soil invertebrates but are difficult to track because for earthworms to regulate essential micronutrients like Zn, there must be uptake and elimination to account for changing soil Zn concentrations (Vijver et al., 2003; Vijver et al., 2005a). Besides trouble with heterogeneous soil matrices, the body of literature for uptake and elimination constants of metals in earthworms is lacking. Adding to these data could benefit the study of bioavailability as well as ecological risk assessment of metals in terrestrial systems (van Straalen et al., 2005 and Nahmani et al., 2009). Extensive metal kinetic research in earthworms, including Zn, performed by Nahmani et al., 2009 illustrated the difficulty in achieving usable elimination data for modeling purposes. They reiterate the need to fill the lacking data set in literature and could only partially fulfill their purposed research due to weight loss from stress on *E. fetida* during metal exposure in contaminated soil. It is recognized that Zn is an essential micronutrient for earthworms and they regulate it carefully making it difficult to discern patterns of uptake and elimination from total body burden measurements in contaminated soils. The need for laboratory controlled soils and

suitable metal exposure levels to maintain fitness, and therefore accurate uptake and elimination mechanisms, is apparent.

A study by Hu et al., 2010 exposed *E. fetida* to TiO_2 and ZnO NPs. Results suggested that when exposed to these metal nanomaterials above 1.0 g kg^{-1}, *E. fetida* experienced DNA damage and mitochondrial abnormalities. These data suggest that even if organisms are able to accumulate and clear metal NPs successfully, there may be potential for delayed genetic damage. Particle size and interaction with soil chemistry has been linked to the success of uptake and retention in earthworms. Nanoparticles have a high affinity for agglomeration in soil pore water resulting in larger particle size, and if NP agglomerates surpass 100 nm they will be less likely to be retained in tissues than a particle <10 nm (Unrine et al., 2008). A study by Kool et al., 2011, found no relationship between toxicity and negative effects on reproduction and particle size when comparing the effect of $ZnCl_2$, bulk ZnO powder, and ZnO NPs on springtails (Collembola). The nano and non-nano ZnO did not differ in their effects on the springtails, but there was a negative affect associated with free Zn^{2+} released from all three forms of Zn. The question that remains is whether Zn NPs are retained in tissues after uptake and elimination.

In order to overcome the challenges associated with evaluating Zn uptake and elimination rates, for this investigation, we used isotopically-labeled ^{68}ZnO nanoparticles to track the fate of NPs in soil and the potential for trophic transfer using the earthworm *Eisenia fetida*. *E. fetida* is a standard organism for soil risk assessment and has demonstrated excellent survival under the control conditions to be used in this work. Isotopically-labeled NPs are not commercially available and had to be synthesized using

a reflux method modified from Dybowska et al. (2011) and Chieng et al. (2012). The behavior of the ^{68}ZnO NPs were compared with the uptake and elimination rates of non-nano bulk ^{68}ZnO powder and $^{68}ZnCl_2$ to illustrate the behavior of ionic versus particulate (Zn NPs and ZnO powder) forms of zinc in a soil system with *E. fetida*. Each of the treatment soils were amended and then split prior to introducing earthworms. One soil set was kept at constant moisture and temperature for 21 days before introducing earthworms to evaluate the impact of soil ageing on the bioavailability of the different Zn forms. We used this general approach to test the null hypotheses depicted and described in Figure 1.

Materials and methods

Experimental design

Based on the results of the range finding pilot test, adult earthworms of the species *E. fetida* were exposed to an 'optimum' concentration of $^{68}ZnCl_2$, ^{68}ZnO bulk powder, and ^{68}ZnO NPs (148 mg/kg Zn) in freshly spiked and aged top soil. All soils were held at 56% moisture (~50% of the soil WHC) throughout the experiment. Double batches of each form of ^{68}Zn-amended soils were prepared at the start of the experiment. One batch of the spiked soils was used for the "initial" exposure, while the second half of the spiked soils were kept in containers and held at constant moisture and temperature (18° C) for 21 days. The aged soils were manually aerated at each time point of the initial experiment (days 0, 1, 2, 4, 7, 14, and 21). $^{68}ZnCl_2$, ^{68}ZnO bulk powder, and ^{68}ZnO metal strips for nanoparticle synthesis were purchased from Trace Sciences; ^{68}ZnO nanoparticles were synthesized in-house and ^{68}ZnO bulk powder was passed through a 200 µm sieve before amending it to the treatment soil. Five earthworm replicates were used for each of 12 time points (0, 1, 2, 4, 7, 14, 21, 22, 23, 25, 28, 35, and 42) over 42

days between the three ^{68}Zn treatments making a 5 x 12 x 3 design. The first six time points represent the uptake phase and the last six time points represent the elimination phase in a clean soil. All earthworms were housed on racks in a light (12 hour light and 12 hour darkness cycles), temperature (17° C) and humidity (60%) controlled exposure lab. Soil moisture within each tube was controlled and adjusted by weight. After day 21, remaining worms were removed from the spike soil and placed into "clean" soil to allow for elimination of the ^{68}Zn. At each time point, five worms were removed randomly based on tube position. These worms were treated as described previously and processed as described in the **Sample Analysis and Quality Assurance/Quality Control Protocols** section below.

The same experiment was repeated with new adult *E. fetida* earthworms in the 21 day-aged ^{68}Zn spiked soils. Earthworm treatment and analysis was identical to the initial experiment.

Experimental methods

All three Zn treatments (bulk powder, NP and, salt) contain isotopically labeled zinc in order to track the fate of zinc in the system. The stable isotope ^{68}Zn was used as a tracer in all of the laboratory experiments. This isotope is well suited for isotope dilution work given its low abundance in nature, availability and, low cost in a high purity form (99.4% ^{68}Zn) available from multiple commercial sources. The range of ^{68}Zn/^{66}Zn in nature is very limited and is generally within the uncertainty of our measurement method (Cloquet et al., 2008). A typical natural ratio for ^{68}Zn/^{66}Zn is ~0.67 while the ^{68}Zn/^{66}Zn ratio of the spike is ~12,000. This large range in isotopic composition will allow for the use of the spike in low masses at relevant concentrations. All bulk tissue and soil

isotopic ratio measurements were made in the Urban Environmental Biogeochemistry Laboratory (UEBL) at Towson University on a Thermo Elemental PQ ExCell ICP-MS. Initial measurements were made on soils, earthworms and ^{68}Zn spike solutions across a range of concentrations to confirm our ability to make accurate and precise measurements of $^{68}Zn/^{66}Zn$; this work has been published as Rodgers et al., 2011. The uncertainty on these measurements has consistently been at better than +/- 1%. This precision allowed us to discern small changes in the isotopic composition during experiments.

Changes in tissue isotope ratios over the course of the experiments were compared to the measured initial isotopic composition of the earthworms at the start of the experiment. Tissue isotope ratios from the zinc salt and bulk ZnO powder treatments were expected to initially increase and eventually return to the background Zn isotope level because those Zn forms were predicted to be the most available during the uptake period and the most readily exchanged from tissues during the elimination period (Figure 1). The NP treatment ratios are of particular interest because of the uncertainty associated with an earthworm's ability to take up particulate NP ZnO as opposed to ionic Zn derived from ZnO NP dissolution. If the Zn isotope ratio in worms from the nanoparticle treatment returns to background levels during the elimination period, then it can be assumed that earthworms do not bioaccumulate zinc nanoparticles, in the lab or in nature, and there is a low potential for trophic transfer. If, however, results show elevated ratios in the nanoparticle treatment organisms after the depuration period as compared with the salt and bulk zinc treatments, then zinc nanoparticles are likely being incorporated into tissues and/or organs in the particulate form that is not readily eliminated and ZnO NPs can be expected to enter the terrestrial food chain.

Sample Analysis and Quality Assurance/Quality Control Protocols

The trace metal content of experimental soils was determined using EPA method 3050B, Acid digestions of sediments, sludges and soils by ICP-MS. This method is designed to quantify the non-silicate environmentally available trace metal concentrations of the experimental soils using a 7 N HNO_3 extraction. The Zn isotope measurements were made on this same extraction by ICP-MS following the procedure described by Rodgers et al. (2011). In addition, soils were analyzed for pH by suspending the samples in E-pure water and utilizing a pH meter.

Earthworms from laboratory experiments were euthanized in ethanol and briefly preserved in formaldehyde prior to dissection and analysis (as in Lev et al., 2010). All earthworms were dissected to remove the gut content from rest of the body tissue. Tissue samples for trace metal and isotope analysis were digested in a 55 mL Teflon vials on a Mars 6 microwave at 150°C for 24 hours using 7 N HNO_3. Analysis of tissue samples was done by ICP-MS.

Chemistry and analysis

Earthworm digestions from the definitive experiments were performed in Teflon tubes with a Mars 6 microwave digestion system using 10 mL 7N HNO_3. Acid extractable and total digestions of all of the Zn amended soil were performed using the same materials as in the earthworm acid digestions (hotplate digestion during pilot tests and Mars 6 microwave during definitive experiments). All digested samples were diluted to a 2% HNO_3 solution with and internal standard prior to analysis by ICP-MS.

All samples from pilot tests and definitive experiments were analyzed by inductively coupled plasma mass spectrometry (ICP-MS) (Thermo Elemental PQ Excell)

as described by Camoponelli et al., 2009. For quality assurance purposes, a method blank and a standard reference material (NIST SRM 2977, mussel tissue) were digested and analyzed with each batch of samples; recoveries for SRM 2977 were within 85% to 105% of the certified values. Mass bias in the isotopic ratio measurements was corrected using a mass bias factor based on repeated measurements of a 30 ppb ^{68}Zn stable isotope standard (SPEX Certiprep), with a known ratio throughout each block of analyzed samples (see Rodgers et al., 2011 for details). The ^{68}Zn stable isotope standard was measured before and after each ICP-MS run, and after every five samples throughout the run.

^{68}ZnO nanoparticle synthesis

Synthesis of ^{68}ZnO NPs was done in-house and used a new method following on methods from Dybowska et. al., 2011 and Cheing et. al., 2012. ^{68}ZnO metal strips (97.8% ^{68}Zn) were purchased from Trace Sciences. Using the Mars 6 microwave system, 700 mg of ^{68}Zn metal particles were added to 10 mL acetic acid (trace metal grade) in MARSXpress Teflon tubes. Tubes were held at 200°C for 15 minutes with a 20 minute ramp time. Isotopically-enriched zinc acetate ((^{68}ZnAc)$_2$) was produced as a white precipitate. This material was separated from the remaining metal by ethanol dissolution and taken to dryness. The (^{68}ZnAc)$_2$ was then dissolved in ethylene glycol (EG; 1M) by reflux at 160°C. After 20 minutes of reflux, the (^{68}ZnAc)$_2$ began going into solution and was completely dissolved after five minutes. After complete dissolution, the mixture immediately began precipitating milky white ^{68}ZnO NPs and the reaction was allowed to continue for another ten minutes. After removal from the heat and time to cool to room temperature, this solution was centrifuged (30 minutes at 4000 rpm) then diluted with

ethanol to separate the NPs from the supernatant (3 hours at 4000 rpm). The NP pellet was dried in an oven overnight (96% recovery).

Characterization

^{68}ZnO NPs were characterized using transmission electron microscopy (TEM) and dynamic light scattering (DLS) at Johns Hopkins University in order to establish the size range and measure the zeta potential. The samples for TEM were prepared using Cu mesh formvar and Cu-coated grids that were freshly glow discharged. ^{68}ZnO NPs were added in a 1:1 deionized water solution, sonicated for 30 minutes, and serial diluted to 100:1. This solution was negative stained using 2% Uranyl Acetate and dried onto the Cu grid. Images were located and generated using an FEI Tecnai 12 transmission electron microscope at 100 kV with an SIS Megaview detector. Figures 8 and 9 show the distribution of ^{68}ZnO nanoparticle size from TEM analysis and the breakdown of point count data (N=8.42 nm (± 3.26)).

For DLS analysis, a suspension of ^{68}ZnO NPs was made by adding 10 mg of ZnO powder to 50 mL of deionized water and sonicating for five hours (see Figure 8). This suspension was then removed, allowed to cool, and centrifuged at 1000 rpm for five minutes. The suspension was removed and stored in a base-cleaned 20 mL vial. A 1 mL sample was placed into a disposable plastic cuvette for particle size analysis. DLS measurements were performed at 24°C using a Malvern ZetaSizer Nano-ZS. A He-Ne laser (633nm) operating at 5mW, probed the ZnO NPs and non-invasive backscatter measurements were recorded. Effective hydrodynamic diameter results modeled by the Stokes-Einstein relationship were the average of five separate measurements, each measurement consisting of 10-15 scans. Another 1 mL of ZnO NPs suspension was

inserted into a clear disposable folded capillary zeta cell. Laser Doppler Micro-electrophoresis is used to measure the zeta potential of a given particle suspension. An interferometric technique measures the velocity of a particle, enabling the calculation of electrophoretic mobility and then zeta potential using the Smoluchowski model. Results were the average of five separate measurements; each measurement consisting of 10-15 scans.

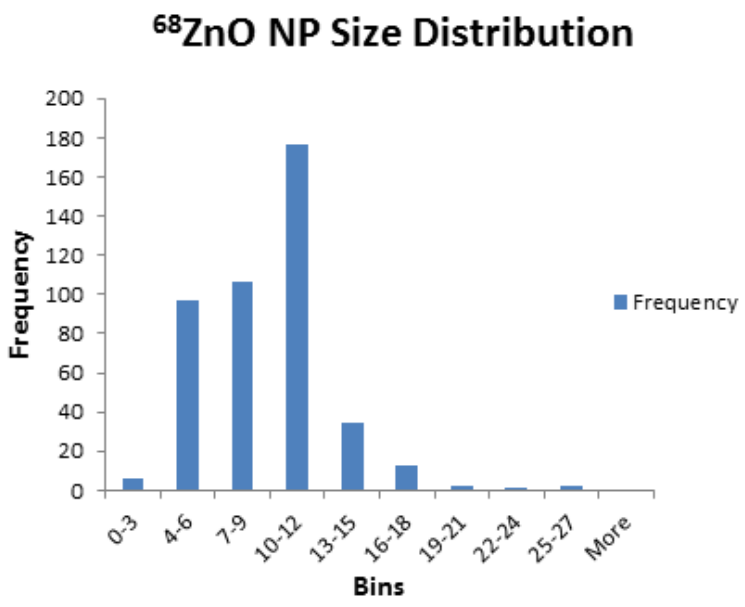
Results

^{68}ZnO NP synthesis results

The ^{68}ZnO NPs characterized via TEM at Johns Hopkins University were measured manually across their diameters to generate point count data (see Figure 8). This allowed us to accurately characterize the mean particle size and confirm that NPs truly fit within the nanoparticle range (<100nm). The particle size distribution of the particles generated for the definitive experiments are shown in Figure 8. The highest frequency of particles were characterized within the 10-12 nm size range, with 7-9 and 4-6 size ranges following in highest frequency respectively with no particles over 30 nm. 438 particles were counted and measured with an average a particle size of 8.42 nm (± 3.26). This anaylysis confirmed a particle size range of 1-30 nm which is well within the nanoparticle range. The photomicrographs in Figure 9 illustrate their uniform, spheroidal structure.

Dynamic light scattering (DLS) analysis was performed on the ^{68}ZnO NPs at Johns Hopkins University in another effort to confirm particle size. The resulting data confirmed a high rate of agglomeration despite excessive sonication before-hand, so the particle size measurements are not reflective of individual nanoparticles seen in the TEM

analysis (236.5 nm ± 0.6573). Zeta potential analysis was also perfomed on the same ^{68}ZnO NPs sample and similarly refelects the zeta potential of the agglomerate surfaces and not the individual particles (21.4 mV ± 0.965).



Bins	Frequency
0-3	6
4-6	97
7-9	106
10-12	177
13-15	34
16-18	13
19-21	2
22-24	1
25-27	2
More	0

Figure 7. Histogram and point count data generated from TEM particle size measurements showing the size distribution for ^{68}ZnO NPs produced at Towson University.

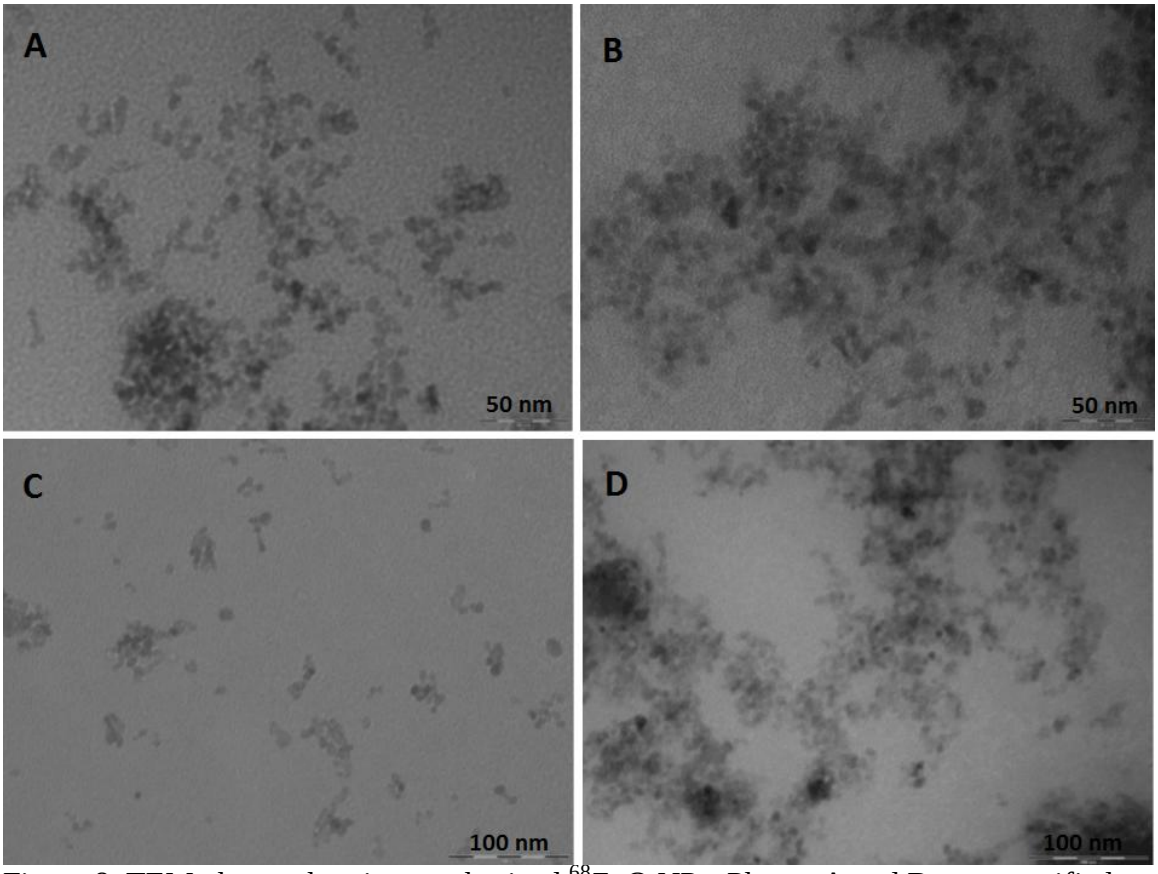


Figure 8. TEM photos showing synthesized ^{68}ZnO NPs. Photos A and B are magnified 455,000x while C and D are magnified 300,000x.

Uptake and elimination

Over the duration of both 42 day experiments, the earthworms remained healthy without manual feeding. Only 1.7% of the 420 specimens did not survive the experiment. Uptake is recorded using the ^{68}Zn/^{66}Zn ratio and demonstrates the replacement of natural ^{66}Zn with the spiked ^{68}Zn added to the soil in the experiment. The results of the Zn uptake analysis showed that the $ZnCl_2$ and NP treatments were quite similar (Fig. 4). The Bulk treatment showed a lower plateau in the isotopic ratio than the other two forms of Zn. This probably occurred due to the bulk powder having a higher surface area since it is a larger particle (>200 nm) than the nano form of ZnO, and

therefore resulting in lower bioavailability to *E. fetida*. Both the uptake and the elimination phases appear to exhibit two compartments within the earthworm – this is perhaps more apparent in the elimination phase. During the elimination phase, a static phase was detected between the "fast" and "slow" compartments. The fast compartment seemed to occur over just one day of exposure to clean soil.

The average Zn concentrations and standard deviations for all treatments of Zn through both experiments are presented in Table 2. Overall, Zn body burden remained relatively constant and at about the same level across all three forms of Zn despite the observed changes in the isotopic ratio. ^{68}ZnO bulk was found at the lowest levels of the three Zn treatments within *E. fetida* in both the initial and aged experiments and of the uptake and elimination values.

Uptake-elimination calculations

Uptake and elimination rates for each treatment were calculated based on a first order kinetic, two compartment model of uptake and elimination following the general method of Spacie and Hamelink (1995). Briefly, the model was:

$$^{68}Zn/^{66}Zn = C_0 + C_x\left[\frac{k_{ufast}+k_{uslow}}{k_{efast}+k_{eslow}}\left(1 - e^{-(k_{efast}+k_{eslow})t}\right)\right] \qquad (2)$$

where:

C_0	Average ^{68}Zn metal concentration in time 0 earthworms (mg/kg)
C_x	Nominal ^{68}Zn spike into soils (mg/kg)
k_{ufast}	Uptake rate in fast compartment (day^{-1})
k_{uslow}	Uptake rate in slow compartment (day^{-1})
k_{efast}	Elimination rate in fast compartment (day^{-1})
k_{eslow}	Elimination rate in slow compartment (day^{-1})
t	time (days)

A first order kinetic model was chosen for this analysis because the rates of uptake and elimination of Zn are a function of soil ratios (C_x). Conversely, a zero order kinetic model

would generate a rate independent of environmental concentrations (Wust et al., 1999). Hypothesis 1 and 2 were evaluated using fully factorial balanced ANOVA (Table 2). The three treatments (ZnO NPs, ZnO bulk, and $ZnCl_2$) were crossed with the two ageing periods (0 day and 42 day). Normality of the data was evaluated using the Shapiro-Wilk test and homogeneity of variance with F_{max} test. The Shapriro-Wilk analysis determined that there was no deviance from a normal distribution of residuals within the elimination coefficients in definitive experiments ($p>0.05$). The plots for uptake and elimination were generated using the R Studio statistical package.

Table 2. ANOVA model results for uptake rates in the Initial (I) and Aged (A) experiments.

		Model fit				**Shapiro-Wilk test**	
Addition	**Coefficient**	**Estimate**	**SE**	**t**	**P**	**W**	**P**
I $ZnCl_2$	K_{ufast}	0.3759	0.0454	8.273	< 0.001	0.9770	0.676
	K_{uslow}	0.0233	0.0053	4.374	< 0.001		
I Bulk	K_{ufast}	0.0943	0.0285	3.31	0.0025	0.9370	0.068
	K_{uslow}	0.0205	0.0040	5.163	< 0.001		
I NPs	K_{ufast}	0.2673	0.0502	5.324	< 0.001	0.9643	0.340
	K_{uslow}	0.1326	0.0220	6.031	< 0.001		
A $ZnCl_2$	K_{ufast}	0.4314	0.0245	17.619	< 0.001	0.8321	0.000
	K_{uslow}	0.0208	0.0025	8.363	< 0.001		
A Bulk	K_{ufast}	0.4068	0.0614	6.63	< 0.001	0.9364	0.059
	K_{uslow}	0.0164	0.0048	3.439	0.00173		
A NPs	K_{ufast}	0.3400	0.0625	5.443	< 0.001	0.8707	0.001
	K_{uslow}	0.1546	0.0243	6.363	< 0.001		

Soils and background measurements

All test soils maintained a pH ranging from 7.4-7.9 before earthworms were exposed and at all times in both experiments. Soil analysis through acid extraction determined that the $^{68}Zn/^{66}Zn$ ratio after being spiked was approximately 4.5. This is well above the measured background ratio for natural Zn. Each experiment, initial and aged, began with sacrificing and analyzing five "control" worms for each of the three Zn types, totaling 30 control subjects. The measured background for control worms was 0.96 +/- 0.01 creating an "in-house" background level used as the initial earthworm isotopic composition. This ratio was reproducible across all batches of earthworms.

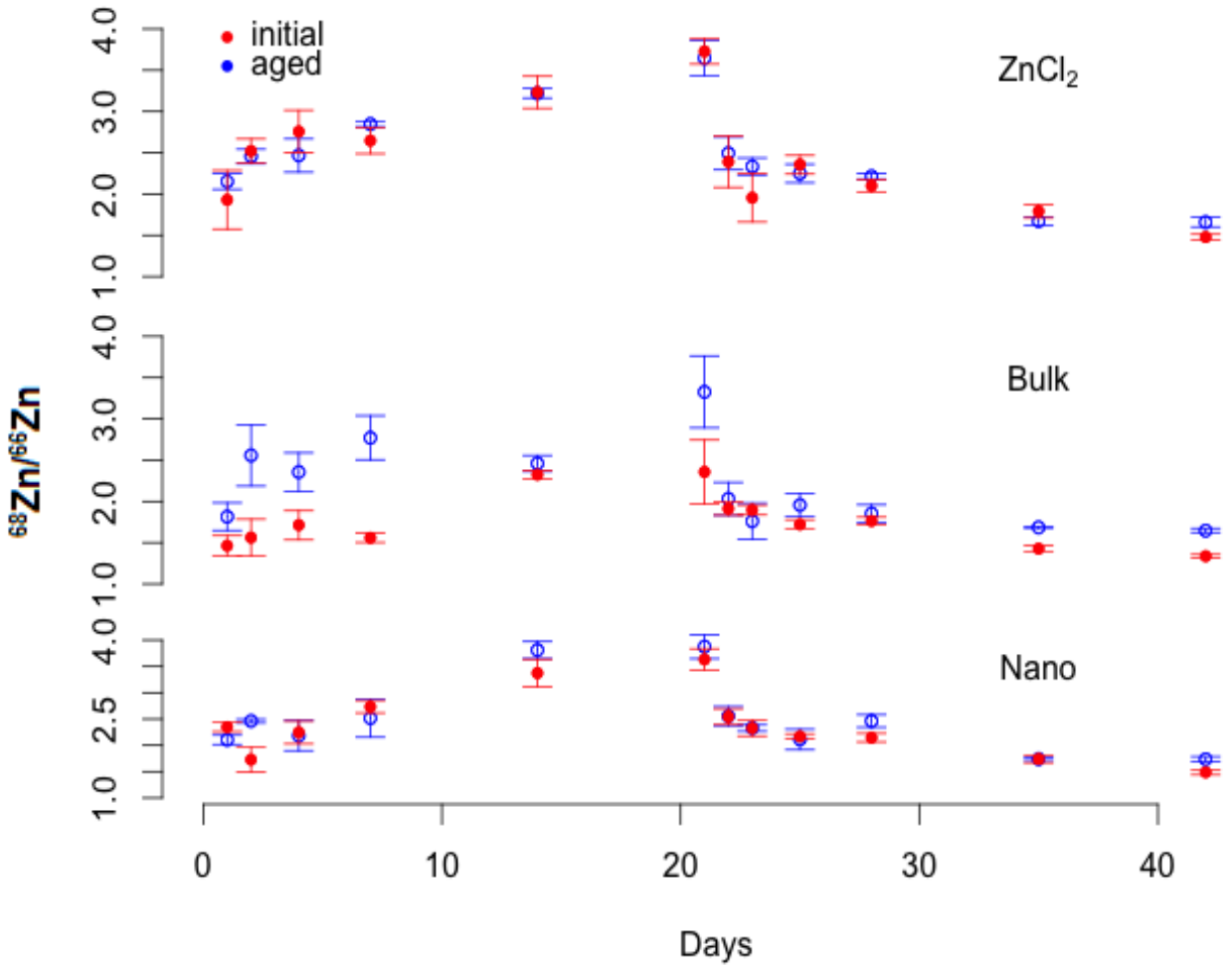


Figure 9. Uptake and elimination kinetics illustrating all three Zn treatments from both Initial (red) and Aged (blue) experiments.

Table 3. Average ^{68}Zn concentration including (± standard deviation) for the raw body burden data in both experiments and all three Zn treatments. I = Initial Experiment, A = Aged Experiment.

Treatment, Experiment	**^{68}Zn Conc. Uptake (mg/kg)**	**Min – Max (mg/kg)**	**^{68}Zn Conc. Elim. (mg/kg)**	**Min – Max (mg/kg)**
$ZnCl_2$ I	194 (±78.8)	52.32-338	208 (±66.8)	48.6-308
$ZnCl_2$ A	183 (±75.4)	29.9-394	175 (±53.4)	66.1-293
Bulk I	167 (±92.8)	43.9-509	176 (±55.9)	43.9-293
Bulk A	164 (±62.3)	52.5-270	139 (±63.2)	23.8-266
NPs I	211 (±76.2)	39.4-358	200 (±68.9)	27.5-358
NPs A	177 (±69.6)	42.9-312	195 (±98.9)	52-663
Time 0 Worms I	48.0 (±12.8)	37-64.3	---	---
Time 0 Worms A	67.4 (±12.2)	51.9-79	---	---

Discussion

Uptake and elimination kinetics

The data demonstrated a clear, yet uncharacteristic, two-compartment model for earthworm uptake and elimination for each of the three Zn forms and between both experiments. Table 3 lists the kinetic rates for the fast and slow compartments for all treatments. The "fast" compartment appears to be a result of the immediate entrance of Zn into the earthworm, likely via the dermis, indicating a high ionic bioavailability. The "slow" compartment may have resulted from an additional pool of Zn that is less bioavailable (form, surface area, biotic ligand binding, etc.) and has been incorporated within biological molecules or chemically altered within the worm and is slowly sloughing off into ionic, and therefore, available Zn. In the case of ZnCl2 and bulk ZnO, perhaps the ionic form of Zn was able to move in and out of the "slow" compartment without being retained, but the high kinetic rates of the NP treatment allude to particle

incorporation. Bioavailability is related to, among other things, particle size and form. This experiment compares ionic zinc ($ZnCl_2$) to non-ionic and particulate zinc (ZnO) as well as addressing particle size differences in the ZnO source. The bulk ZnO (>200 nm) treatment resulted in earthworms maxing out at a lower $^{68}Zn/^{66}Zn$ than the other two treatments but notably, the ZnO NP (<30nm) treatment. These data support the idea that a lower surface area on a larger particle results in less bioavailability to an organism. The static phase illustrated in Figures 12 and 14 could be a result of dynamics within each organism. Perhaps the worms were stressed when initially entering the "clean" soil starting the elimination phase, and it caused them to excrete or stop processing soil for a short time until they acclimated to their new environment. There also seems to be behavioral variability among the worms that could account for the limited resolution in the models. It appears that the regulation of Zn occurs in elimination, not uptake, as excretion and Zn flow between compartments occurs during depuration.

The data suggest that compartment 1 and compartment 2 within an earthworm comprise different tissues and therefore perform different functions in essential micronutrient use and transport. Compartment 1 most likely consists of organ tissues and/or rapid uptake within the gut. This is shown in Figures 11-14 as the duration of compartment 1 lasts about one day. A similar study investigating the compartmentalization of ^{68}Zn found that of the three main body tissues in an earthworm (dermis, organs, and gut), the organs were able to equilibrate with ^{68}Zn spiked soil most quickly (Rodgers et al., 2011). In this same study, the two-day elimination of ^{68}Zn from *Lumbricus terrestris*'s organs indicates that the organs in earthworms are sensitive to micronutrient regulation and a compartment that experiences rapid turnover. Vijver et al.,

2005 also saw fast turnover rates when *L. rubellus* in spiked ^{65}Zn soil reached a constant level after only a few hours of being moved into "clean" soil. Conversely, compartment 2 is probably a combination of gut tissue and dermis making it more difficult for Zn to equilibrate and thereby termed the "slow" compartment.

Focusing on uptake in the Initial experiment (Figure 11), $ZnCl_2$ and ZnO NPs showed no significant difference in uptake between the two rates in compartment 1. This suggests that either the small size of the NPs or rapid dissolution of the NPs make this phase indistinguishable from free ionic Zn and quickly enters through the dermis or become immediately bioavailable to the worm when entering the gut tissue. The Bulk treatment shows significantly less uptake in compartment one presumably because the ZnO bulk particles are larger and have a lower surface area than the ZnO NP treatments, causing a slower rate and less uptake overall. In comparing the Initial uptake rates with the Aged uptake rates (Figure 13), there is a difference in compartment 1: all three Zn treatments were not significantly different from one another. In an aged soil situation, it is likely that the ZnO bulk particles were able to weather and release more ionic Zn, therefore allowing the worms to more easily take up Zn through the dermis and gut since they have become more bioavailable than in freshly-spiked soil. The "slow" compartment, compartment 2, in both the Initial and Aged experiments depicted a significantly different relationship that separates the ZnO NPs from the ZnO bulk and $ZnCl_2$ treatments. In both experiments, the "slow" second compartment in the NP treatment was faster than the other two treatments. This could be accounted for by a unique interaction with nano-particulate Zn: some nanoparticles moved through the cell membrane and remained intact, with some of those particles dissociating ionic Zn within

the compartment. Or some of the Zn from the NPs was dissolved quickly outside of the compartment, and was able to pass through the cellular membrane channels ionically.

Figures 11 and 14 illustrate *E. fetida*'s ability to eliminate and turn over ^{68}Zn the longer the organism is contained in "clean" soil. In both experiments, the elimination curves were not statistically different between treatments and soil type (Initial and Aged). This was to be expected if Zn that has been stored within the worm's tissues was in an easily regulated form (e.g. non-NP form). Particle size likewise did not have an effect on elimination, because the bulk and NP treatments behaved similarly. The tails of the elimination models do not, however, completely reach the level of the control organisms indicating that a small amount of spiked ^{68}Zn is still being retained in the tissues. These data coincide with Vijver et al. (2005) as they also found that *L. rubellus* still retain ^{65}Zn after 32 days of elimination. Before plotting, the data were examined and 17 out of 420 worms were removed from the data set either due to death or those that were living but seemed to not have performed metabolic activity based on the ^{68}Zn/^{66}Zn ratio. The data that was removed comprised only about 4% of the overall data collected.

Sensitivity of approach

Upon seeing the resulting fast and slow elimination compartments and observing a very sudden (1 day) fast compartment, it would have been helpful to assign hourly earthworm removal and analysis to gain a clearer picture of the physiology associated with Zn elimination. The data recovered using a typical uptake and elimination experimental design left one value to be modeled in each of the three Zn treatments. This "fast" elimination compartment resulted in somewhat unreliable uptake data. Unfortunately, there does not appear to be any published earthworm uptake and

elimination data in which the first model in a two-compartment model occurred so rapidly. Similarly, taking soil samples and pH readings at every time point could have shed more light on the mechanisms that impact Zn metal uptake, elimination, and sequestration. Nahmani et al., 2009 published uptake and elimination rates for Zn by *E. fetida* exposed to multiple contaminated field soil samples. To more accurately compare their data with ours, the aged experiment's uptake rates are compared since they didn't freshly spike any soils for exposure. Our average uptake data obtained between all three Zn treatments using a two compartment model is 0.457 while Nahmani et al., 2009's is 0.0327 using a one compartment model (from bulk concentration k_{1soil}, not including their pore water calculations). Our methods of exposing *E. fetida* to Zn resulted in higher kinetic rates for uptake. Perhaps this was due to our method of amending a fixed amount of isotopically-labelled ^{68}Zn into soil under laboratory conditions instead of exposing *E. fetida* to multiple field-contaminated soils. Being able to isotopically trace the amended Zn through *E. fetida* could have resulted in more accurate uptake rate constants than simply measuring the body burden of worms exposed to contaminated soil. Had we not used isotopically-labeled ^{68}Zn, our uptake rates would most likely have been lower and shown minimal change as it would be difficult to distinguish our amended Zn from background Zn in the earthworm or in the soil. Since Nahmani's uptake rates were lower than ours, perhaps this is a reflection of method choice. Or a combined measurement of all three forms of Zn treatments averaged together rather than comparing a specific form or particle size fraction of Zn to Nahmani's study. Nahmani et al., 2009 followed a 42 day uptake exposure with no depuration phase into clean soil, as their study focused on predicting steady state tissue metal concentrations. Therefore, our elimination constants

from a two compartment model are not comparable with their excretion constants obtained using a one compartment model.

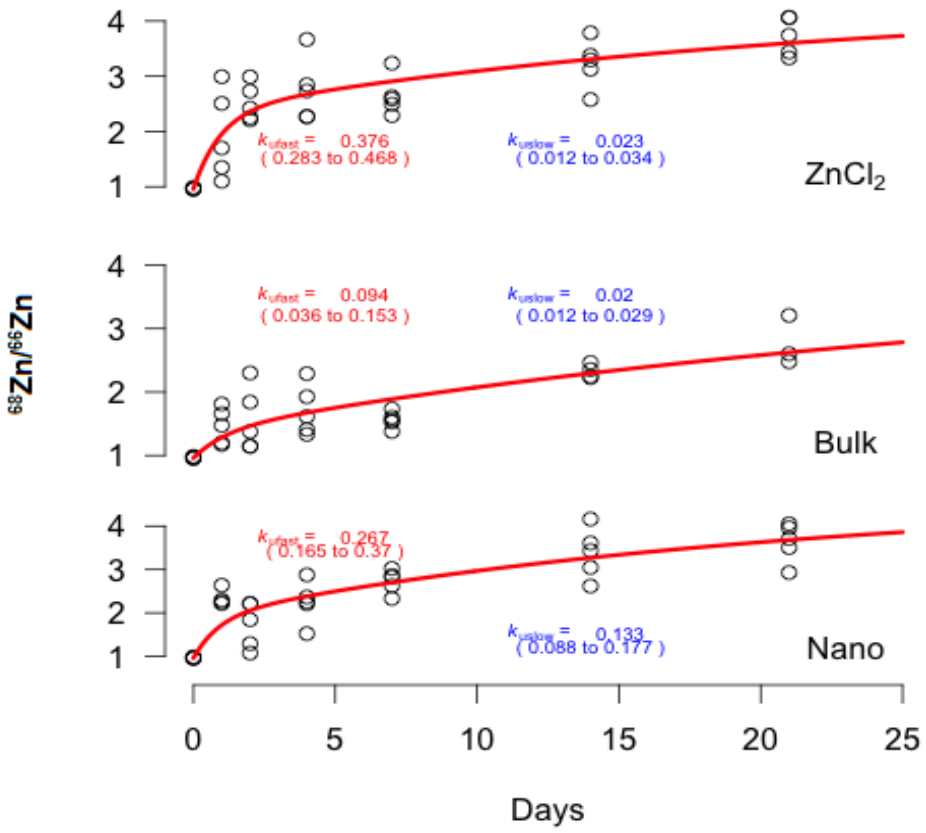


Figure 10. Uptake kinetics for each of the three Zn treatments ($ZnCl_2$, Bulk ZnO, and ZnO NPs) in the Initial exposure. Fast and slow uptake constants with their respective 95% CI ranges are also listed.

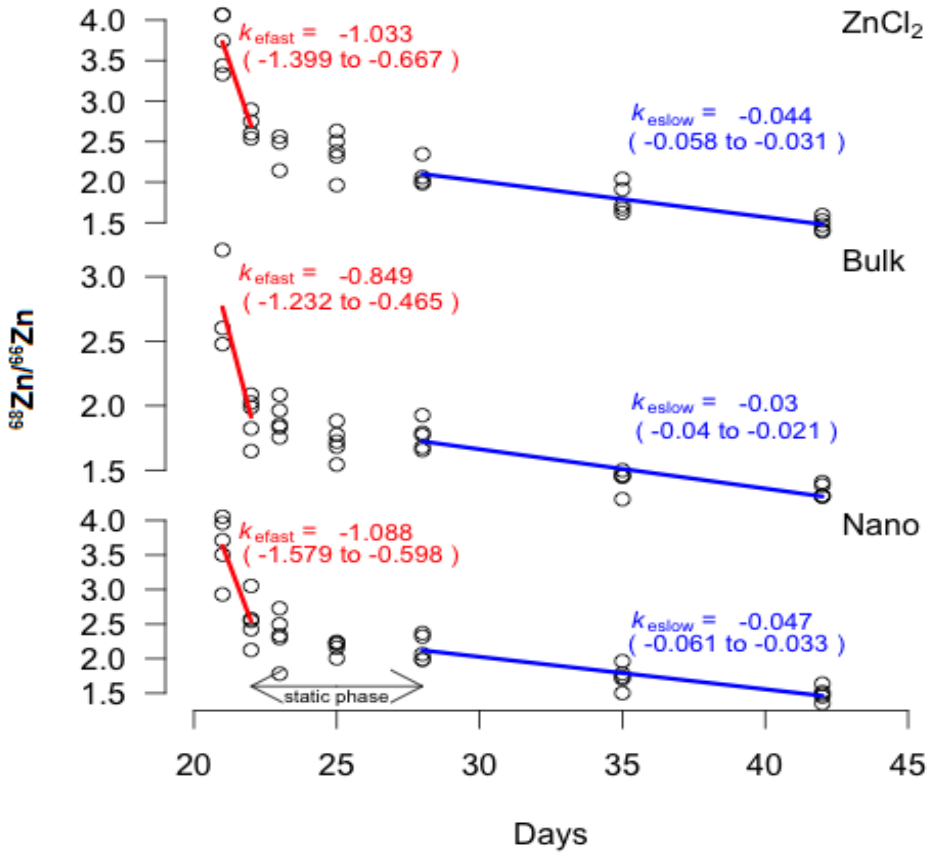


Figure 11. Elimination kinetics showing a two compartment model with a static phase between the fast and slow compartments in all three Zn treatments ($ZnCl_2$, ZnO Bulk, and ZnO NPs) in the Initial exposure experiment. Fast and slow elimination constants with their respective 95% CI ranges are also listed.

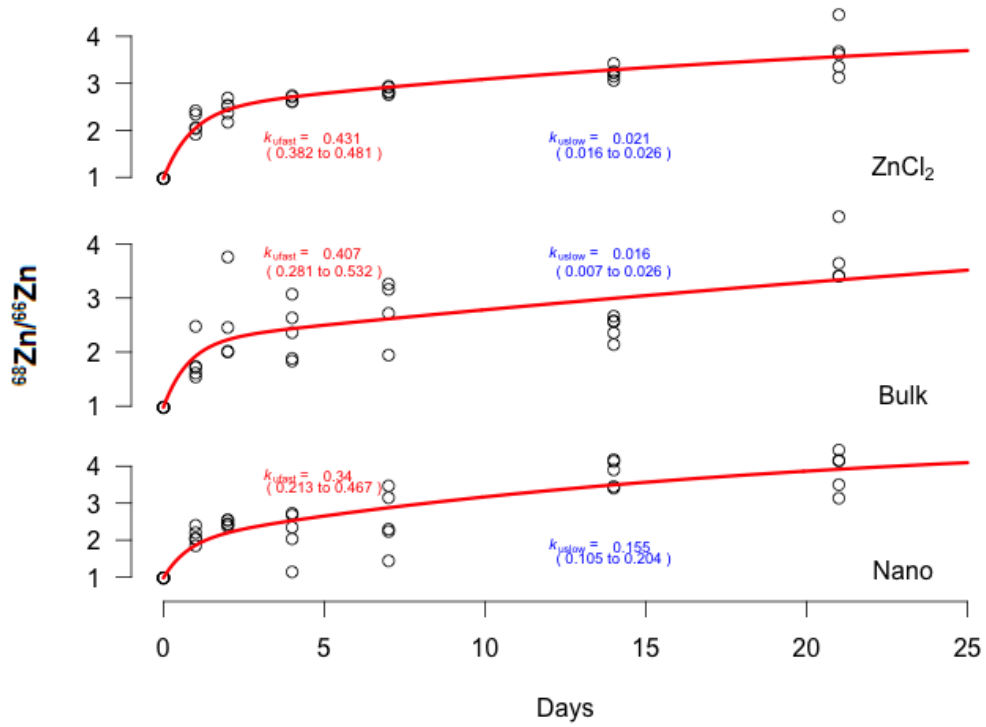


Figure 12. Uptake kinetics comparing the $^{68}Zn/^{66}Zn$ ratio in the three Zn treatments ($ZnCl_2$, Bulk ZnO, and ZnO NPs) in the Aged exposure. Fast and slow uptake constants with their respective 95% CI ranges are also listed.

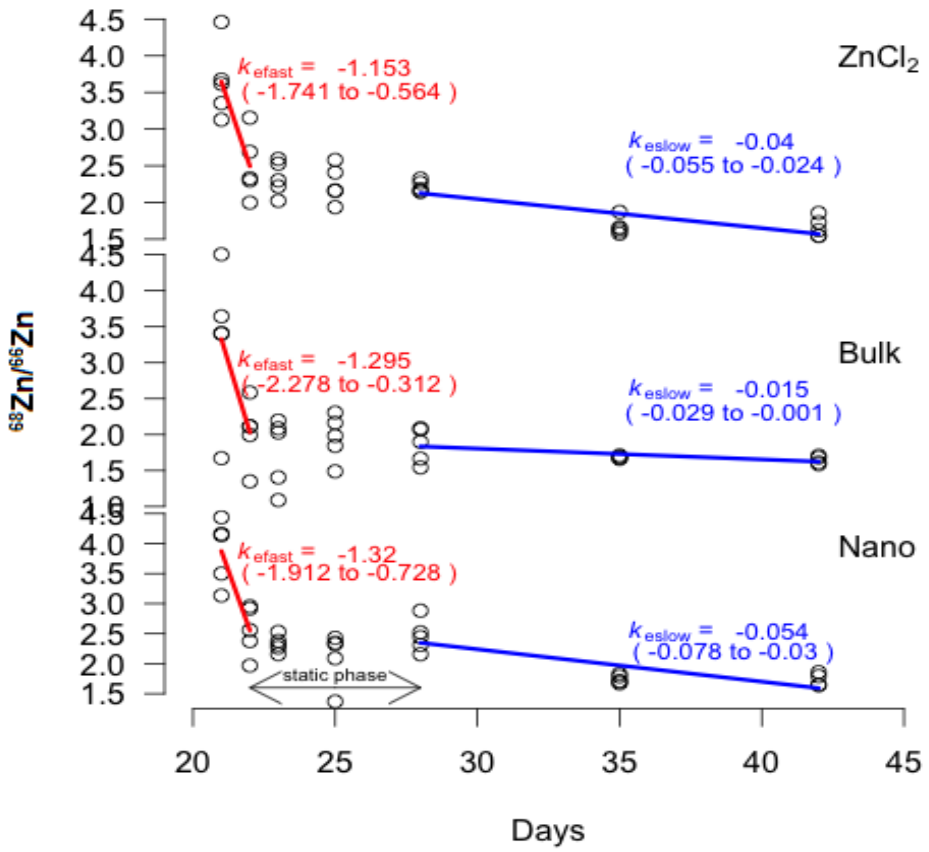


Figure 13. Elimination kinetics showing a two-compartment model with a static phase between the fast and slow compartments in all three Zn treatments ($ZnCl_2$, ZnO Bulk, and ZnO NPs) in the Aged exposure experiment. Fast and slow elimination constants with their respective 95% CI ranges are also listed.

Table 4. The rate constants for the fast and slow compartments of the kinetic models for all three treatments of Zn in the Initial and Aged experiments. U= uptake data, E= elimination data.

	K_{fast}	Min – Max	K_{slow}	Min – Max
INITIAL				
$ZnCl_2$ U	0.376	0.283-0.468	0.023	0.012-0.034
Bulk U	0.094	0.036-0.153	0.02	0.012-0.029
NPs U	0.267	0.165-0.37	0.133	0.088-0.177
$ZnCl_2$ E	1.033	1.399-0.667	0.044	0.058-0.031
Bulk E	0.849	1.232-0.465	0.03	0.04-0.021
NPs E	1.088	1.579-0.598	0.047	0.061-0.033
AGED				
$ZnCl_2$ U	0.431	0.382-0.481	0.021	0.016-0.026
Bulk U	0.407	0.281-0.532	0.016	0.007-0.026
NPs U	0.34	0.213-0.467	0.155	0.105-0.204
$ZnCl_2$ E	1.153	1.741-0.564	0.04	0.055-0.024
Bulk E	1.295	2.278-0.312	0.015	0.029-0.001
NPs E	1.32	1.912-0.728	0.054	0.078-0.03

Conclusions

The results from this experiment require revision of our original two-tailed hypotheses. Hypothesis 1 was most correct in that the $^{68}Zn/^{66}Zn$ isotope ratio in *E. fetida* was identical for elimination between all three Zn treatments. The uptake rates were slightly different in that, the bulk ZnO showed a significantly slower uptake rate in the fast compartment (0.094) than $ZnCl_2$ and ZnO NPs (0.376 and 0.267 respectively). ZnO NPs showed a higher rate of uptake in the second compartment (0.133) than $ZnCl_2$ and bulk ZnO (0.023 and 0.02 respectively). Hypothesis 2 predicted no change in $^{68}Zn/^{66}Zn$ isotope ratio from the initial experiment to the aged experiment, and was also rejected. The uptake data from the aged experiment showed that the bulk ZnO treatment (0.407) no longer differed from $ZnCl_2$ or ZnO NPs in the first compartment (0.431 and 0.34

respectively). ZnO NPs still displayed a higher uptake rate (0.155) than $ZnCl_2$ and bulk ZnO in the second compartment (0.021 and 0.016 respectively), however, and elimination rates for all three Zn treatments were still not significantly different from one another.

In addition to evaluating Zn uptake and elimination rates from isotopically-labeled Zn, we investigated the potential uptake and retention of ZnO nanoparticles that might lead to movement up a terrestrial food chain. Our data indicates that ZnO NPs of this particle size (<50 nm) are not retained in earthworm tissue to a greater extent than other forms of Zn and therefore do not pose additional risk for trophic transfer. Earthworms exhibited a nearly uniform Zn body burden across all $ZnCl_2$ and ZnO NPs exposures. This result suggests very little difference in the behavior of these two forms of Zn and is therefore useful for the purpose of risk assessment. The results of this investigation suggest that the behavior of ionic $ZnCl_2$ can be used to model the behavior of ZnO nanoparticles in soil systems. ZnO NP's fate in the environment is tied to size, agglomeration, and soil chemistry which should also be considered when evaluating site specific risk. Through DLS and TEM analysis, we were able to see the immediate agglomeration of NPs prior to amending soils. Had these NPs been coated for consumer use, a manufacturer could have added a coating perhaps consisting of a surfactant or tannic acid, which would potentially lower agglomeration or stabilize the NP respectively (Judy et al., 2011). Knowing this and seeing the rapid uptake and elimination of ZnO NPs in *E. fetida* before and after soil weathering, leads us to conclude that agglomeration may

inhibit the uptake and retention of manufactured metal NPs but, they may still affect terrestrial invertebrates (i.e. sludge application) by contributing to the bioavailable pool in contaminated soils.

Appendices

Table 5. Summary data for $ZnCl_2$ treatment during pilot tests.

Nominal (ppm)	Soil Actual (ppm)	Avg. Zn Body Burden (ppm)	Avg. Weight Change (g)	Survivability (%)
0	107.1	244.9	0.03088	100
100	95.7	459.8	-0.01837	100
200	361.8	334.1	-0.01037	83
500	164.5	437.1	0.03007	100
900	238.3	875.0	0.03764	83
1000	296.5	704.8	0.008113	100
1200	393.9	908.4	0.02877	83
1400	404.5	809.1	-0.002414	83
1600	508.4	690.5	-0.006406	83
1800	692.1	1078	0.01922	100
2000	1068	1094	0.009808	100
3000	1796	2217	-0.01316	100
4000	2436	1964	-0.04697	50
5000	2413	807.6	-0.1235	17

Table 6. Summary data for ZnO Bulk treatment during pilot tests.

Nominal (ppm)	Soil Actual (ppm)	Avg. Zn Body Burden (ppm)	Avg. Weight Change (g)	Survivability (%)
0	123.9	292.6	0.0924	83
100	140.6	230.8	0.06223	100
200	213.1	368.2	0.08808	100
500	389	572.4	0.08692	100
900	560.7	998.3	0.06995	100
1000	633.1	915.6	0.0832	100
1200	982.6	1481	0.08807	100
1400	1029	1384	0.07982	100
1600	1182	1504	0.02662	100
1800	1014	1467	0.06938	100
2000	1123	1944	0.04415	100
3000	1784	2648	0.04438	67
4000	2970	3068	0.03667	100
5000	2996	3218	0.06668	100

Table 7. Summary data for ZnO NPs treatment during pilot tests.

Nominal (ppm)	Soil Actual (ppm)	Avg. Zn Body Burden (ppm)	Avg. Weight Change (g)	Survivability (%)
0	23.06	260.5	0.002833	100
100	92.4	256.0	-0.02096	100
200	161.4	228.9	0.01987	100
500	371.4	500.8	0.01828	100
900	721.2	739.8	0.03197	100
1000	860.1	854.3	0.02832	100
1200	403.5	732.9	0.0273	100
1400	1524	1212	0.03718	100
1600	1306	959.4	0.0295	100
1800	1089	652.3	0.03427	100
2000	1988	756.4	0.00948	83
3000	2734	1143	0.01615	100
4000	3522	2792	0.04022	100
5000	3789	2815	0.005633	50

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